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Activation of Silent *gal* Genes in the *lac-gal* Regulon of *Streptococcus thermophilus*

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Streptococcus thermophilus **strain CNRZ 302 is unable to ferment galactose, neither that generated intracellularly by lactose hydrolysis nor the free sugar. Nevertheless, sequence analysis and complementation studies with *Escherichia coli* demonstrated that strain CNRZ 302 contained structurally intact genes for the Leloir pathway enzymes. These were organized into an operon in the order *galKTE*, which was preceded by a divergently transcribed regulator gene, *galR*, and followed by a *galM* gene and the lactose operon *lacSZ*. Results of Northern blot analysis showed that the structural *gal* genes were transcribed weakly, and only in medium containing lactose, by strain CNRZ 302. However, in a spontaneous galactose-fermenting mutant, designated NZ302G, the *galKTE* genes were well expressed in cells grown on lactose or galactose. In both CNRZ 302 and the Gal$^+$ mutant NZ302G, the transcription of the *galR* gene was induced by growth on lactose. Disruption of *galR* indicated that it functioned as a transcriptional activator of both the *gal* and *lac* operons while negatively regulating its own expression. Sequence analysis of the *gal* promoter regions of NZ302G and nine other independently isolated Gal$^+$ mutants of CNRZ 302 revealed mutations at three positions in the *galK* promoter region, which included substitutions at positions −9 and −15 as well as a single-base-pair insertion at position −37 with respect to the main transcription initiation point. Galactokinase activity measurements and analysis of *gusA* reporter gene fusions in strains containing the mutated promoters suggested that they were *gal* promoter-up mutations. We propose that poor expression of the *gal* genes in the galactose-negative *S. thermophilus* CNRZ 302 is caused by naturally occurring mutations in the *galK* promoter.**

After its discovery almost 40 years ago, the *Escherichia coli* lactose operon, encoding enzymes of lactose metabolism, became the first model for gene regulation (reviewed in reference 4). The key component of this system is the *lac* repressor (LacI), the product of the *lacI* gene. The *lac* operon contains a primary operator (O_1), which is the major element of repression by LacI, and two pseudo-operators, which enhance repressor binding to O_1 by cooperativity. Control of the *lac* operon also involves activation by the cyclic AMP receptor protein. Many other paradigm systems of negative control have since been described, including GalR, one of the two repressors of the *gal* regulon encoding enzymes of galactose transport and metabolism in *E. coli*. Regulation of the *gal* regulon is mediated through GalR, GalS (Gal isorepressor), and the cyclic AMP receptor protein. GalR and GalS negatively regulate transcription of the two promoters of the *gal* operon, although GalS is not as efficient as GalR (57).

The bioconversion of lactose, which is the primary carbon and energy source in milk, into lactic acid is an essential process in industrial dairy fermentations carried out by lactic acid bacteria. Genetic studies of the metabolic pathways for lactose utilization in these gram-positive bacteria have revealed a variety of *lac* operons that differ from the paradigm known in *E. coli* (13). The thermophilic yogurt bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus* contain a highly homologous *lacSZ* operon in which the β-galactosidase (*lacZ*) gene is located downstream from the *lacS* gene encoding a lactose permease (LacS), which belongs to the galactoside-pentose-hexuronide translocators (27, 41, 48, 49).

Although lactose is efficiently transported and hydrolyzed intracellularly, many strains of *S. thermophilus* and *L. bulgaricus* do not grow on galactose and ferment only the glucose portion of lactose, while the galactose is excreted into the medium in amounts stoichiometric with the uptake of lactose (20, 22). Kinetic studies indicated that LacS mediates both galactoside exchange (e.g., lactose-galactose) and movement of galactosides and protons (15). The exchange reaction is highly favored with excess galactosides on either side of the membrane and may account for the galactose-negative (Gal$^-$) phenotype of *S. thermophilus* in milk which contains an excess of lactose (40). Another explanation for the Gal$^-$ phenotype may be the absence of functional Leloir pathway enzymes, including galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT), and UDPglucose 4-epimerase (GalE), products of the *galK, galT*, and *galE* genes, respectively. Remarkably, under appropriate selective conditions, such as limiting lactose and excess galactose, Gal$^+$ derivatives of *S. thermophilus* were obtained which fermented galactose and contained Leloir enzyme activities (21, 50). However, no

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Relevant features	Reference or source
E. coli strains		
HB101	F$^-$ *hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44* λ^-	46
LE392	F$^-$ *hsdR514*(r_K^- m_K^+) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ^-	46
TG1	Δ*(lac-pro) supE thi hsdD5* F′[*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	46
S. thermophilus strains		
CNRZ 302	Wild-type Gal$^-$ strain	5
ST11	Gal$^-$ strain	32
NZ302G	Gal$^+$ class I mutant	This study
SS1	Gal$^+$ class I mutant	This study
SS2	Gal$^+$ class II mutant	This study
SS3	Gal$^+$ class I mutant	This study
SS4	Gal$^+$ class I mutant	This study
SS5	Gal$^+$ class II mutant	This study
SS6	Gal$^+$ class I mutant	This study
SS7	Gal$^+$ class III mutant	This study
SS8	Gal$^+$ class I mutant	This study
SS9	Gal$^+$ class II mutant	This study
NZ302GΔR	NZ302G carrying a disruption in *galR*	This study
ST11ΔR	ST11 carrying a disruption in *galR*	This study
Plasmids		
pACYC184	Tc^r	9
pUC19	Ap^r	Gibco-BRL
pNZ680	4.9-kb *gal* insert in pACYC184; Tc^r	This study
pNZ273	Contains *gusA* reporter gene; Cm^r	39
pNZ6871	Contains *galR* gene, and *galK* promoter of strain CNRZ 302 fused to *gusA*; Cm^r	This study
pNZ6872	Contains *galR* gene, and *galK* promoter of strain SS2 fused to *gusA*; Cm^r	This study
pG$^+$host9	Temperature-sensitive shuttle vector; Em^r	28
pNZ684	pG$^+$host9 with internal fragment of *galR* gene; Em^r	This study
pNZ6811	Derived from pNZ6871, carries *galR* gene of strain CNRZ 302; Cm^r	This study
Phage		
M13mp18/19		58

molecular explanation was given, and the genetics of the Leloir pathway has only been poorly investigated in *S. thermophilus*. The *lacSZ* operon of strain A147 was found to be preceded by *galE* and *galM* (42). The *galM* gene appeared to be constitutively expressed and could encode a mutarotase that, similar to the homologous enzyme of *E. coli*, forms the galactokinase substrate α-D-galactose from β-D-galactose pyranose generated from lactose by β-galactosidase (LacZ) (7). However, *S. thermophilus* A147 is not a Gal$^+$ strain.

The present study was undertaken to gain insight into the presence and regulation of the *gal* genes of *S. thermophilus* and the mechanism by which the genes, in particular the *galK* gene, are prevented from being expressed. Here we describe the characterization of the *gal* operon, consisting of the *galK, galT* and *galE* genes, and its promoter from *S. thermophilus* CNRZ 302, for which galactose-fermenting (Gal$^+$) revertants have been reported (5). A regulatory gene, *galR*, was identified that is divergently transcribed from the *gal* operon. Analysis of mRNA for the *gal* metabolic genes from a Gal$^+$ fermenting derivative of CNRZ 302 indicated that regulation occurred at the transcriptional level. In contrast, the *gal* metabolic genes of the original Gal$^-$ strain were not sufficiently transcribed to allow galactose metabolism. Furthermore, we demonstrate that GalR acts as a transcriptional activator of both the *gal* and *lac* operons and negatively regulates its own expression. To the best of our knowledge, this is the first report describing the mechanisms regulating galactose utilization in *S. thermophilus* at the molecular level.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *S. thermophilus* strains were subcultured in M17 broth (Oxoid, Basingstoke, England), containing either 1% lactose, glucose, or galactose as necessary, at 42°C unless stated otherwise. The taxonomic position of strain CNRZ 302 was confirmed by 16S rRNA sequence analysis and corresponded to *Streptococcus thermophilus* (GenBank accession number X68418). *E. coli* strains HB101 or LE392 and TG1 were used for the isolation of pACYC184- and pUC19-derived plasmids and for the propagation of bacteriophage M13 chimeras, respectively. *E. coli* was routinely grown in TY medium (45) or brain heart infusion (Difco) broth with aeration at 37°C. MacConkey agar base (Difco Laboratories) supplemented with 1% galactose was used to detect galactose-positive (Gal$^+$) *E. coli* strains. Agar media were prepared by adding 1.5% agar to broth. The antibiotics used for selection in media were chloramphenicol at 4 μg/ml and erythromycin at 2.5 μg/ml for *S. thermophilus* and chloramphenicol at 15 μg/ml, tetracycline at 12 μg/ml, and ampicillin at 100 μg/ml for *E. coli*. Em^r *E. coli* strains were selected on brain heart infusion agar containing 150 μg of erythromycin per ml.

DNA isolation and manipulations. Transformation and isolation of plasmid DNA from *E. coli* were performed essentially by established protocols (46). Chromosomal DNA was extracted from exponentially growing cells of *S. thermophilus* by the procedure of Hayes et al. (19). The Anderson and McKay (3) lysis procedure was used to detect plasmid DNA in *S. thermophilus*. Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were used as recommended by the supplier (Gibco-BRL, Life Technologies, Gaithersburg, Md.). DNA fragments were recovered from agarose gels with the GlassMatrix DNA isolation system (Gibco-BRL). Electroporation of *S. thermophilus* was performed by the procedure of Mollet et al. (32) with the modification that the harvested cells were incubated in the electroporation buffer at 4°C for at least 4 h prior to electroporation. PCR was performed under the conditions described previously (25) using *Taq* polymerse (Life Technologies) or *Pwo* polymerase (Boehringer Mannheim). Oligonucleotides were synthesized by Eurogentech (Gent, Belgium).

Cloning of *gal* genes. *S. thermophilus* CNRZ 302 total genomic DNA was digested with *Eco*RI, and the DNA fragments were separated by agarose gel electrophoresis (0.7% agarose). The DNA was transferred to a GeneScreen Plus (Dupont, Boston, Mass.) membrane by established methods (46). The membrane was hybridized with a 700-bp *Acc*I fragment, containing part of the *S. thermophilus* F140 *galK* gene kindly provided by B. Hutkins (34). The labeling of this fragment with horseradish peroxidase and the hybridization and detection methods were as described in the manufacturer's manual for the ECL system (Amersham, Little Chalfont, United Kingdom). Fragments of approximately 5 kb were recovered, ligated with *Eco*RI-linearized calf intestinal alkaline phosphatase-treated pACYC184, and used to transform *E. coli* HB101 and LE392. Gal^+ clones were selected as red Tc^r colonies on McConkey galactose agar at a frequency of approximately 1%. Analysis of the plasmid content of all 10 Gal^+ colonies indicated that they contained a recombinant plasmid with a 4.9-kb insert that showed an identical restriction pattern. One of the clones, designated HB101(pNZ680), was used in further experiments.

Nucleotide sequence analysis. DNA fragments were subcloned in the phage vectors M13mp18 and M13mp19 with TG1 as a host by using standard techniques (46). Nucleotide sequences of both strands were determined by the dideoxy-chain termination method (47), adapted for Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) with either the M13 universal primer or specifically synthesized primers. The *gal* promoter regions of *S. thermophilus* CNRZ 302 and its 10 Gal^+ derivatives were isolated as 350-bp PCR fragments from agarose gels. The purified fragments and primers were annealed by boiling for 5 min and rapidly freezing in liquid nitrogen, and sequencing proceeded as described above. The sequence data were assembled and analyzed with PC/GENE version 6.6 (Genofit, Geneva, Switzerland). Amino acid sequence comparisons were performed with the EMBL (release 31.0), SwissProt (release 28.0), and NBRF/PIR (release 40.0) databases using the FASTA program (36), through the facilities of the CAOS/CAMM Center (Nijmegen, The Netherlands). The curvature of DNA was predicted as described by Munteau et al. (33).

Isolation of Gal^+ *S. thermophilus* strains. *S. thermophilus* CNRZ 302 cultures grown in M17 broth supplemented with 1% lactose were diluted 100-fold into M17 broth containing 1% galactose and 0.01% glucose and incubated for 24 h. Cultures that exhibited growth were transferred to M17 broth containing 1% galactose (Gal-M17) and incubated for 16 to 20 h. Ten Gal^+ single-colony isolates were obtained by plating 10 cultures treated as described above on M17 agar with 1% galactose, and these were designated NZ302G and SS1 to SS9.

RNA isolation, Northern blotting, and primer extension analysis. *S. thermophilus* strains were grown in M17 broth (50 ml) containing 1% lactose, glucose, or galactose to an optical density (600 nm) of 0.6 to 1.0. Total RNA was isolated from the harvested cells as described by Kuipers et al. (26) with the following modification: before being subjected to bead beating, the cells were treated with 2 mg of lysozyme per ml for 2 min on ice, which increased the RNA yield. The RNA was either fractionated on a 1.0% formaldehyde gel (46) or glyoxylated and fractionated on a 1.2% agarose gel as described previously (52). RNA size markers were obtained from Bethesda Research Laboratories. RNA was transferred to GeneScreen Plus membranes by following the protocols outlined by the manufacturers. Hybridizations were performed at 65°C in a 0.5 M sodium phosphate buffer (pH 7.2) containing 1.0% bovine serum albumin (fraction V), 1.0 mM EDTA, and 7.0% sodium dodecyl sulfate, and the blots were washed at 55 to 65°C in 1.0 to 0.1× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% sodium dodecyl sulfate (46).

Gel-purified restriction fragments and PCR products that had been labeled by nick translation with α-^{32}P (Amersham) were used as hybridization probes (46). These included a *galK*-specific probe isolated as a 0.6-kb *Hpa*I-*Hind*III fragment from pNZ680, a *galTE* probe consisting of a 1.2-kb *Pst*I-*Eco*RI fragment, a *galR*-specific probe amplified from pNZ680 with primers 5′-GCC CAA TGA GTA GGC C-3′ and 5′-CGG ATA TTA ACT ATC GCT G-3′, and a 1.6-kb *lacS*-specific probe generated with primers 5′-TAA CAC AGG TGA TCC AAA GCA-3′ and 5′-GGT GAC CAG AAC TCA AGA AG-3′. The primer GALRAS (5′-GTT GAA ATA GAT ACA CCT GC-3′), which is complementary to the 5′ end of the sense strand of the *galR* gene, was end labeled with γ-^{32}P using polynucleotide kinase (Bethesda Research Laboratories).

Primer extension was performed by annealing 5 ng of oligonucleotide 3′-ACT AAC CAC TCG TAT GCC TGA T-5′ and 5 ng of oligonucleotide 5′-GTA TCC TCT GTT ACG G-3′ complementary to the mRNA for *galK* and *galR*, respectively, to 20 μg of *S. thermophilus* RNA and performing complementary DNA synthesis as previously described (52). The reaction products were separated by electrophoresis on a 5% sequencing gel, together with a sequencing reaction product obtained using the same primers.

Construction of plasmids for analysis of *galK* promoters. The promoters from the CNRZ 302 and a class II Gal^+ mutant, strain SS2, were amplified by PCR using primers 5′-CGG GAT CCT GCT AAT TTT GCG ATA TCT G-3′ and 5′-CGG AAT TCC TTT AAA CTT TTC TCT TAA C-3′, with built-in *Bam*HI and *Eco*RI sites (underlined), respectively. The 210-bp products were cloned into *Bam*HI-*Eco*RI-digested pUC19, generating pNZ680.1 and pNZ680.2. A 1.5-kb *Nsi*I-*Eco*RV fragment from pNZ680 containing the CNRZ 302 *galR* gene and a potential transcription terminator (Fig. 1A) was attached in frame to the *galR* promoters (this step was necessary for the stability of further constructs), using the *Pst*I and *Eco*RV sites in the pUC19 derivatives, generating plasmids pNZ6861 and pNZ6862. The "*gal* promoter and *galR* gene cassettes" were removed as 1.7-kb *Eco*RI-*Hin*dIII fragments (the 3′ recessed terminus of the *Hin*dIII sites were first filled) and subsequently ligated into the *Eco*RI-*Sca*I-digested pNZ273. Plasmid pNZ273 contains the *gusA* reporter gene that encodes the β-glucuronidase enzyme. The plasmids, designated pNZ6871 and pNZ6872 for the CNRZ 302 and SS2 mutants, respectively, contain the *galK* promoter fused to the *gusA* gene and the *galR* gene under its own promoter. The integrity of the amplified promoter regions was confirmed by sequence analysis. The constructs were initially made in *E. coli* and were subsequently used to transform *S. thermophilus* ST11 and selected on M17 agar containing 1% sucrose and chloramphenicol. Histochemical screening for β-glucuronidase activity by selecting for blue colonies with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (Research Organics Inc., Cleveland, Ohio) was performed as previously described (11).

Construction and use of integrating and complementing plasmids. A 750-bp *Pml*I-*Nla*IV fragment of the *galR* gene from pNZ680 was ligated into the calf intestinal alkaline phosphatase-treated *Eco*RV site of the thermosensitive pG^+host9 vector, generating pNZ684 (Fig. 1C). Electrotransformation of pNZ684 into *S. thermophilus* NZ302G resulted in four Em^r transformants, all of which contained the expected plasmid at 30°C. To obtain integration of pNZ684, cultures grown overnight in M17 sucrose broth with erythromycin at 28°C were diluted 100-fold into fresh medium and reincubated at 28°C to allow the exponential phase of growth to resume. The cultures were shifted to 42°C and grown until they reached stationary phase. Dilutions of the cultures were plated at 42°C, and integrants appeared as Em^r colonies after 24 to 48 h of incubation. Correct integration within the *galR* gene in the chromosome (Fig. 1D) was confirmed by both Southern hybridization and PCR for integrant NZ302GΔR (data not shown). The *galR* gene of strain ST11 was disrupted in the same manner.

To construct a plasmid with the *galR* gene without *gusA*, the *gusA* gene in pNZ6871 was removed by digesting with *Eco*RI-*Hin*dIII and the recessed 3′ termini of the remaining 4.9-kb fragment were filled in and ligated to generate pNZ6811.

Enzyme and protein assays and chromatography. The *S. thermophilus* strains were grown in M17 broth containing either 1% lactose, galactose, glucose, or sucrose with the appropriate antibiotics to an optical density at 600 nm (OD_{600}) of 1.0. For the preparation of extracts, cells were disrupted with zirconium glass beads in a Bead Beater (Biospec Products, Bartlesville, Okla.) for 3-min treatments with intervals of 1 min on ice between treatments and cellular debris was removed by centrifugation. The extracts were kept on ice, and enzyme assays were performed within 4 h. Galactose 1-phosphate uridylyltransferase activity was assayed in the resultant extracts with 30 to 350 μg of protein per assay by the spectrophotometric method of Isselbacher (23). β-Galactosidase was assayed at 37°C by the method of Miller (31) using 1 to 6 μg of protein per assay and galactokinase assays by the method of Ajdic et al. (2). All enzyme activity measurements presented were the mean of at least two independent experiments. Proteins concentrations were estimated by a dye binding assay (8).

Lactose and galactose were detected by high-performance liquid chromatography with a refractive index detector (M410; Waters) using a Polyspher CHPb18 column (Merck). The separations were carried out on a M6000 isocratic pumping system (Perkin-Elmer) in combination with an automatic sample injector (717+; Waters), and water was used as the eluent.

Nucleotide sequence accession number. The GenBank accession number assigned to the nucleotide sequence encoding *S. thermophilus galR*, *galK*, *galT*, and the partial *galE* gene is U61402.

RESULTS

Isolation and localization of the *S. thermophilus galK* and *galT* genes. Southern hybridizations of genomic DNA of *S. thermophilus* strain CNRZ 302 identified an *Eco*RI fragment of approximately 5 kb that hybridized with a probe consisting of a 0.7-kb internal fragment of the *galK* gene of *S. thermophilus* F140 (34). A minibank of fragments including the hybridizing

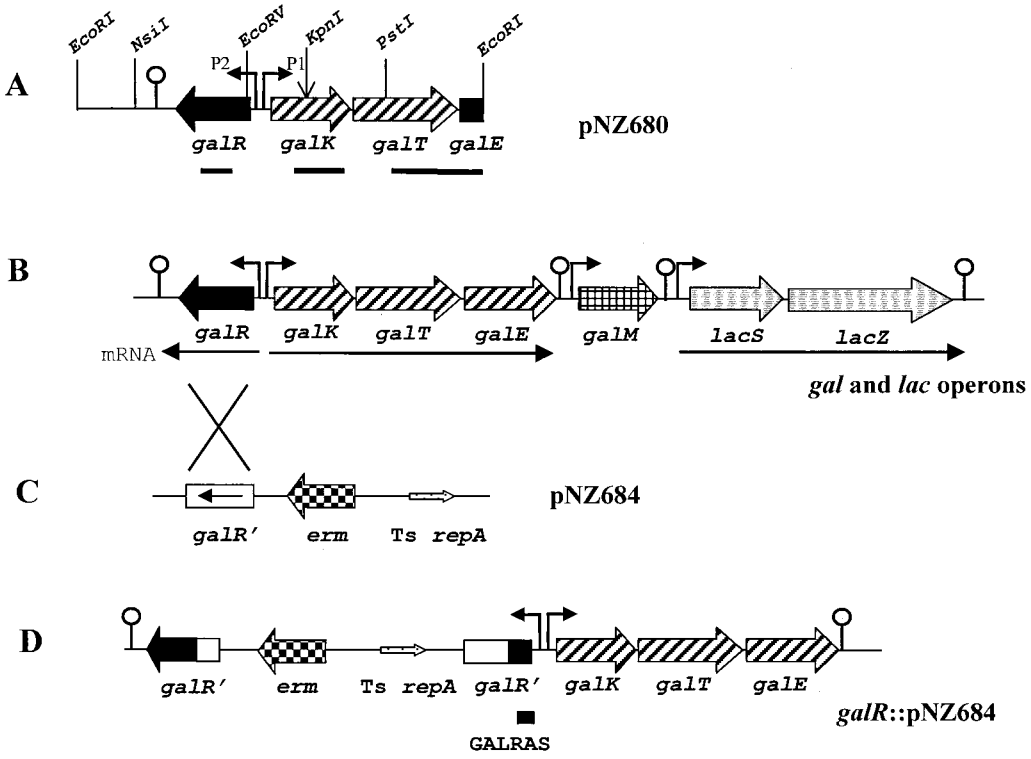


FIG. 1. Organization of the *gal* and *lac* genes in their operons, and construction of a *galR* gene disruption. (A) Illustration of the *gal* genes on the 4.9-kb *Eco*RI fragment of *S. thermophilus* CNRZ 302 cloned in pNZ680. The positions of relevant restriction enzyme cleavage sites are indicated, and the frameshift mutation in the *Kpn*I site is marked with a vertical arrow. The *galK* and *galR* promoters are indicated by arrows P1 and P2, respectively. The black lines below the genes represent the DNA probes used in the Northern blot experiments. (B) Organization of the *gal* and *lac* genes in the chromosome of *S. thermophilus* CNRZ 302. The mRNA transcripts for the *gal* and *lac* genes are indicated by arrows. Positions of promoters and potential terminators are indicated. (C) Plasmid pNZ684 carrying the thermosensitive replicon (Ts *repA*) of pG$^+$host9 (small arrow) is shown in a linear form for convenience; the directions of the sense strand in the 750-bp *Pml*I-*Nla*IV *galR* fragment in pNZ684 and the Em^r gene (*erm*) are indicated. (D) Orientation of integration of pNZ684 in the *galR* gene. The two partially deleted copies of *galR* are designated *galR'*, and the location of the primer GALRAS is indicated by the bar.

fragment was constructed in the chloramphenicol resistance (*cat*) gene of pACYC184, and the putative *galK* gene of *S. thermophilus* CNRZ 302 was isolated by functional complementation of the *galK2* mutation of *E. coli* HB101. The complementing plasmid, designated pNZ680 (Fig. 1A), contained a 4.9-kb insert that allowed HB101 to form red colonies on McConkey galactose agar and utilize galactose as the sole carbon source in minimal M9 medium. Introduction of a frameshift mutation in the *Kpn*I site on pNZ680 resulted in a plasmid which could not restore a Gal$^+$ phenotype to HB101, indicating that the *galK* gene was overlapping this site (data not shown). Moreover, pNZ680 also complemented both the *galK2* and *galT22* mutations of *E. coli* LE392, indicating that it also contained the *galT* gene of *S. thermophilus*. Although these *gal* genes are hardly expressed in *S. thermophilus*, the promoter upstream of the *cat* gene in pACYC184 is likely to be responsible for their expression in *E. coli*. The presence of a functional *galT* gene was further confirmed by assaying for GalT enzyme activity. Cell extracts of LE392(pNZ680) contained 119 nmol of GalT activity per min per mg, whereas no activity was detected for the LE392 strain alone.

Organization and similarity studies of the *S. thermophilus gal* region. Commencing at the *Kpn*I site on pNZ680, nucleotide sequence analysis in both directions revealed the *galK* open reading frame (ORF), 1,164 bp in length (Fig. 1A). The deduced *galK* sequence had the strongest similarity to the GalK proteins of several gram-positive bacterial species including *Streptococcus mutans* (83%) and *Lactobacillus casei* (70%) (2, 6). The *S. thermophilus* CNRZ 302 GalK was also 79% similar to the GalK of the Gal$^+$ *S. thermophilus* F410 strain (34). A potential ribosome binding site (5′-GAGA-3′), complementary to the 3′ end of the 16S rRNA of lactic acid bacteria (12), was located 8 bp upstream from the first translational initiation codon at nucleotide (nt) 1483.

Upstream of *galK* located in a divergent orientation, a 1,014-bp ORF was designated *galR* on the basis of the similarity of its deduced amino acid sequence to proteins of the LacI-GalR family of transcriptional regulators (55) (Fig. 1A). The translational initiation site at nt 1340 is proposed on the basis of the position of the putative ribosome binding site (5′-AGGAGGA-3′, nt 1351 to 1345) and the similarity between related proteins (see also below). The *S. thermophilus* GalR had the greatest similarity to the GalR repressor of *S. mutans* (75%; 57% identity) and the potential GalR repressor of *L. casei* (59%; 40% identity) (2, 6). There was also significant similarity, 53 and 48% (35 and 27% identity), to the evolved β-galactosidase (EbgR) and galactose (GalR) repressors, respectively, of *E. coli* (18, 54). An inverted-repeat structure and a stretch of five T nucleotides (nt 95 to 56) that could

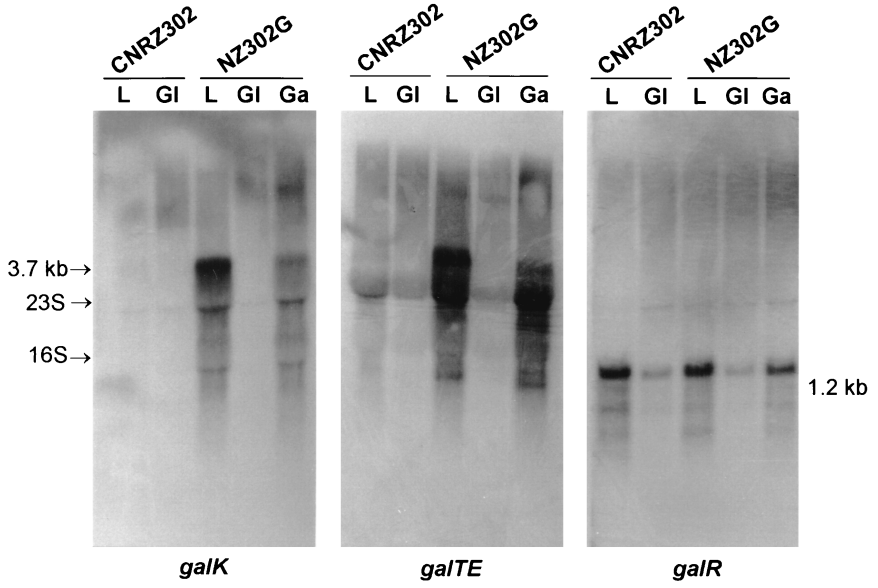


FIG. 2. Northern blot analysis of RNA isolated from *S. thermophilus* strains CNRZ 302 (Gal^-) and NZ302G (Gal^+) grown on glucose (Gl), lactose (L), or galactose (Ga). The probes used for the hybridization are indicated below the blot. The 23S and 16S rRNAs and the mRNA transcript sizes are indicated by arrows.

function as a rho-independent transcriptional terminator (38). followed the *galR* gene sequence.

DNA sequence analysis downstream of *galK* revealed the *galT* gene (1,482 bp), whose deduced sequence was similar (67 to 74%) to the GalT proteins from several gram-positive bacteria including *S. mutans, L. casei*, and *Lactococcus lactis* (Fig. 1A) (2, 6, 53). The stop codon for the *S. thermophilus galK* gene and the start codon of the *galT* gene were separated by just 19 bp. The translational initiation site for *galT* was preceded by a putative ribosome binding site (nt 2654 to 2660). Finally, a fourth ORF was present immediately downstream of the *galT* gene and reading beyond the pNZ680 clone (Fig. 1A). The nucleotide and predicted amino acid sequence were identical to the aminoterminus of the previously characterized *galE* gene from *S. thermophilus* A147 (42).

The organization of *lac* genes in relation to the *galE* gene, which have been characterized for *S. thermophilus* A147 (41, 42), was demonstrated to be identical in CNRZ 302. Long-range PCR was performed using primers based on the sequences of the *galK* gene of CNRZ 302 and the *lacS* gene (41) of A147, which resulted in the expected 5.7-kb product. Restriction enzyme analysis of the PCR product showed an identical pattern to that of A147, confirming the presence of the *galE, galM*, and *lacS* genes downstream of *galK-galT* in strain CNRZ 302 (Fig. 1B).

Transcriptional analysis of the *gal* genes. The transcription of the *gal* genes was analyzed in the wild-type *S. thermophilus* strain CNRZ 302, and in strain NZ302G, an isogenic spontaneous Gal^+ mutant strain. Strain NZ302G has a doubling time of 58 min in M17 medium containing 1% galactose at 42°C, in contrast to the wild-type CNRZ 302 parental strain which does not grow at all on galactose. The Gal^+ phenotype of NZ302G was stably maintained even after several subcultures in M17 containing lactose.

Northern analysis failed to detect hybridization signals for *galK* or *galTE* from the glucose-grown wild-type and Gal^+ strains (Fig. 2). Only after prolonged exposure, were weak signals obtained for lactose-grown wild-type cells (data not shown). However, mRNA was detected for the lactose- and galactose-grown NZ302G cells (Fig. 2), in accordance with the Gal^+ phenotype. The weaker signal obtained for the galactose-grown cells may be due to the poorer-quality RNA obtained as a result of their slower growth. The transcripts were stronger when hybridized with the larger *galTE* probe than when hybridized with the *galK* probe. The size of the predominant mRNA hybridizing to the *galK* and *galTE* probes was approximately 3.7 kb, indicating that the *galK, galT*, and *galE* genes, which are 1.2, 1.4, and 1.0 kb, respectively, are transcribed together as a single mRNA. In conclusion, sufficient induction of *galKTE* mRNA for galactose metabolism occurred only in the Gal^+ NZ302G strain when it was cultured in lactose- or galactose-containing M17 medium.

A major transcript of approximately 1.2 kb was identified for *galR* from both the wild-type strain grown in lactose medium and the mutant strain grown in lactose or galactose medium (Fig. 2). However, only a weak signal for this *galR* mRNA was detected in glucose-grown cells for both strains. The size of the transcript for the 1.0-kb *galR* gene suggests that it is transcribed alone and supports the functional role of the terminator following *galR*. These data indicate that the expression of the *galR* gene is also regulated at the level of transcription.

Mapping and characterization of the *galR* and *galK* promoters. The transcriptional start points of the *galK* and *galR* genes were determined by primer extension analysis. Total RNA was isolated from lactose- and glucose-grown *S. thermophilus* CNRZ 302 cells and from lactose-, glucose-, and galactose-grown NZ302G cells. A major transcriptional start site was observed for the *galK* gene of strain NZ302G that mapped at an A residue (nt 1452), 6 bp downstream of the inferred −10 sequence (TACGAT) (Fig. 3A). The latter was separated by 17

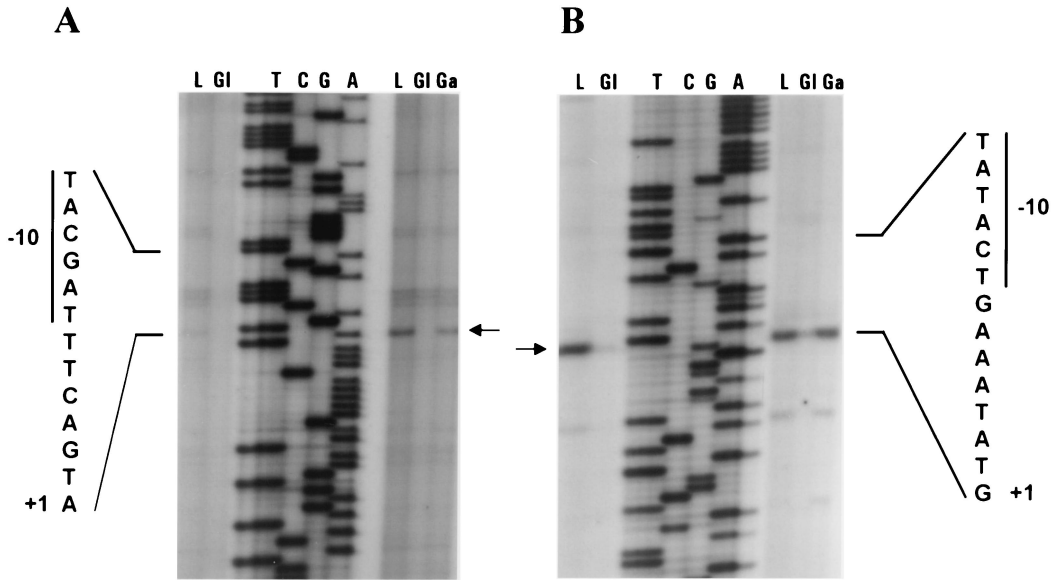


FIG. 3. Primer extension analysis of the 5′ ends of the RNA transcripts for the *galK* (A) and *galR* (B) genes of *S. thermophilus* CNRZ 302 (two left-hand lanes) and NZ302G (three right-hand lanes) grown in lactose (L), glucose (Gl) or galactose (Ga). The ladder obtained from pNZ680 sequenced with the relevant primer is in the middle of each panel. Relevant nucleotide sequences of the promoter regions are presented on either side of the figure. The −10 region is denoted by a vertical bar, and the transcription initiation sites are indicated by +1. The arrows point to the major primer-extended products.

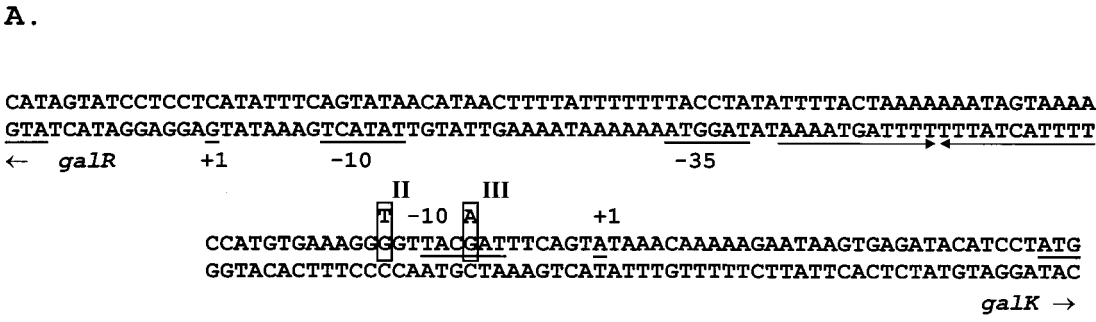
bp from a −35 sequence (TTGATT) that conforms well to the *E. coli* and *S. thermophilus* promoter consensus sequences (Fig. 4A). Essentially no clear signal for the *galK* gene of CNRZ 302 was detected, in accordance with its Gal^- phenotype (Fig. 3A).

For *galR*, a strongly labeled extension product was detected that initiated at a G residue (nt 1352) 7 bp upstream of a putative −10 sequence (TATACT) (Fig. 3B) for both CNRZ 302 and NZ302G. A putative −35 sequence (TAGGTA) could be found 18 bp upstream of the −10 box (Fig. 4A). The presence of the primer extended products for *galK* and *galR* matched exactly the results obtained in the Northern experiments and confirmed control at the transcriptional level.

Effect of the *galR* gene disruption on galactose utilization. To determine the function of the *galR* gene in the Gal^+ *S. thermophilus* NZ302G, the gene was disrupted using pNZ684, consisting of the temperature-sensitive pG^+ host9 vector that carried an internal fragment of the *galR* gene (Fig. 1C). The disruption caused by pNZ684 resulted in two partially deleted copies of the *galR* gene, one of which lacks the DNA for the 17 N-terminal amino acids including most of the conserved DNA binding motif while the other suffers from a 200-bp deletion that can encode 67 amino acids of the C-terminal region (Fig. 1D). In contrast to the NZ302G strain, the isogenic NZ302GΔR integrant could no longer grow in M17 broth containing 1% galactose. To determine whether NZ302GΔR could utilize the galactose moiety of lactose, its growth was compared with that of the parental strain NZ302G in medium containing 0.4% lactose. Although NZ302G is Gal^+, when the strain is grown in medium containing excess lactose (1%), the glucose moiety is preferentially metabolized while galactose is excreted, and presumably acid inhibits growth and prevents subsequent metabolism of the galactose portion of lactose. Reduction of the concentration of lactose to 0.4% eliminates this imbalance while supporting normal growth of strain NZ302G (Table 2). High-performance liquid chromatography analysis of the spent medium indicated that the galactose moiety of lactose was completely metabolized by strain NZ302G while, in contrast, a substantial amount of galactose (72% of the amount that could be hydrolyzed from lactose) was not utilized by the NZ302GΔR integrant. Moreover, the doubling time of NZ302GΔR on lactose increased to approximately 1 h in comparison to that of NZ302G, which is 25 min, and the final OD_{600} was less than half that of NZ302G (Table 2).

To exclude any possible polar effects of the integration of pNZ684 in the NZ302GΔR integrant, complementation of its *galR* mutation was studied. Plasmid pNZ6811 contains the *galR* gene under its own promoter on a high-copy-number vector based on pNZ123 (12). Three transformants of NZ302GΔR harboring pNZ6811 grew to an OD_{600} of approximately 1.4 in M17 medium containing 0.4% lactose broth, and no residual galactose was detected in the cell-free supernatant (Table 2). Furthermore, the transformants could again utilize galactose as the sole carbon source (data not shown). Thus, GalR is necessary for the ability to utilize galactose.

Effect of *galR* disruption on transcription of the *gal* and *lac* genes. Northern hybridizations were performed to determine the effect of the *galR* disruption on the transcription of the *galR* gene and of the *gal* and *lac* operons. The primer GALRAS, which is complementary to the 5′ end of the sense strand of *galR*, was chosen since it can hybridize to the single copy of the 3′-deleted *galR* (*galR′*) in the chromosome that is under control of the *galR* promoter (Fig. 1D). The 1.2-kb *galR* transcript, which was only weakly visible for NZ302G growing on glucose, gave a signal of much greater intensity for strain NZ302GΔR (Fig. 5A). The 5′-deleted copy of *galR* did not appear to be transcribed since the same result was obtained by probing with a 600-bp fragment internal to the *galR* gene (Fig. 5B). This constitutive overexpression of the 3′-truncated *galR′* was also



B.

Microorganism	Gene	Promoter regions
S. thermophilus	*galK*	**TTTTACTA**AAAAAA**TAGTAAAA**ATATTGATT (-35)
S. thermophilus	*lacS*	**TATTA**G**TAAAATTTTA**G**TAAA**AAACACTGAAATTATTGACT (-35)
S. thermophilus	*galR*	**T**AAA**AGT**TATGTTAT**ACT**GAA**A** (-10)
S. mutans	*galK*	A**TTTA**G**TAAAATTTTA**G**TAAA**AATATTGACC (-35)
S. mutans	*galR*	TTTTACTAAAATTTTACTAAAT (-10)

FIG. 4. (A) Intergenic region containing the promoter sequences for the *galK* and *galR* genes. The −10 and −35 regions are underlined, and the transcriptional start sites are indicated as +1. The point mutations for the three classes (I, II, and III) of *S. thermophilus* Gal^+ mutants are as described in the text. Inverted repeats are indicated by arrows. (B) Alignment of similar regions in the promoters of *lac* and/or *gal* genes of *S. thermophilus* and *S. mutans*. Inverted repeats in the *S. thermophilus galK* promoter are indicated by arrows. Nucleotides that are complementary in each arm of the inverted repeats are in boldface type.

observed for lactose-grown cells (data not shown) and suggests that the product of the *galR* gene is a negative regulator of its own expression. It should be noted that termination of transcription of *galR′* in NZ302GΔR is located at an unknown point within the integration plasmid pNZ684, and therefore it is coincidental that the *galR* and *galR′* transcripts appear to have similar sizes.

A *galK* probe was used to monitor the transcription of the *galKTE* operon in NZ302G and the mutant NZ302GΔR (Fig. 5C). While the 3.7-kb transcript of the *galKTE* operon was observed when NZ302G was grown on lactose, no transcript was detected for NZ302GΔR. This indicates that GalR is an activator of transcription for the *galKTE* operon and explains why the galactose moiety of lactose is not effectively metabolized by strain NZ302GΔR.

LacS is the sole galactoside transporting activity in *S. thermophilus* and therefore is essential for both lactose and galactose transport (15). We speculated that GalR may also play a role in the regulation of the *lac* operon (*lacS-lacZ*). Northern hybridization of NZ302G probed with an internal fragment of *lacS* showed that a 5.2-kb transcript, corresponding to the sizes of the *lacS* and *lacZ* genes, was expressed weakly when the strain was grown on glucose (barely visible on the blot) and induced when the strain was grown on lactose or galactose (Fig. 5D). The *lac* operon was still transcribed in NZ302GΔR when the strain was grown on glucose, but there was no longer induction of transcription when it was grown on lactose. This indicates that in addition to being the positive regulator of the *gal* operon, GalR functions as an inducer of transcription of the *lac* operon.

Effect of *galR* disruption on GalT and LacZ enzyme activities. To further substantiate the effect of the *galR* gene disruption on the expression of the *gal* and the *lac* operons, enzyme assays were performed. Since the *galT* gene is the central one of the three genes in the *gal* operon, GalT activity was used as a measure of the activity of the Leloir pathway enzymes (Table 3). In strain NZ302G, GalT activity is induced three- to fourfold in lactose- and galactose-grown cultures in comparison to growth in glucose-containing medium. In contrast, very low activity was detected in the *galR* disruption strain NZ302GΔR when grown on glucose and no significant increase was observed following growth on lactose, indicating a lack of induction of the enzyme. However, the levels of activity of the NZ302GΔR mutant complemented by pNZ6811 were similar

TABLE 2. Growth and galactose utilization of *S. thermophilus* NZ302G and derivatives in medium containing 0.4% lactose

Strain	Final OD_{600}	% Galactose[a]
NZ302G	1.84	ND[b]
NZ302GΔR	0.76	0.143
NZ302GΔR1(pNZ6811)	1.37	ND
NZ302GΔR2(pNZ6811)	1.30	ND
NZ302GΔR3(pNZ6811)	1.43	ND

[a] Percentage of galactose remaining in medium following growth.
[b] ND, not detectable (<0.01%)

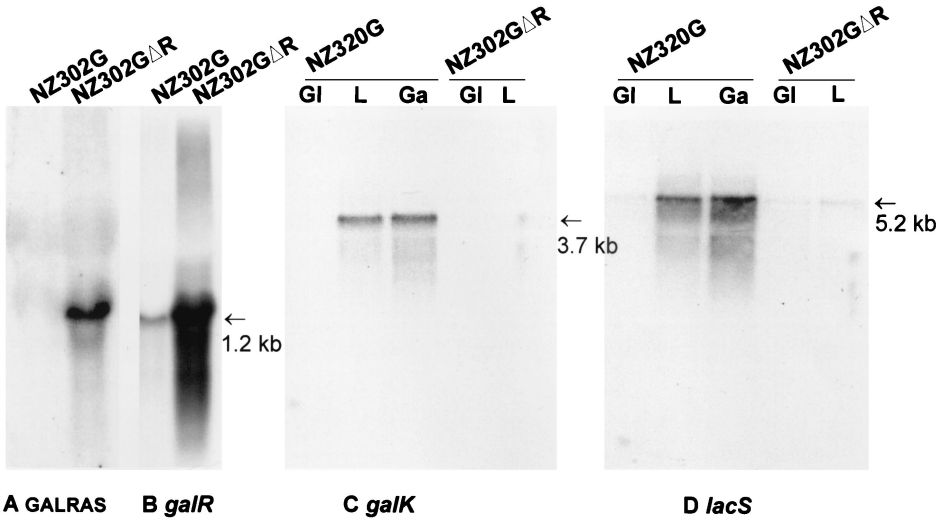


FIG. 5. (A and B) Northern blot analysis of RNA from *S. thermophilus* NZ302G and NZ302GΔR grown on glucose hybridized with the GALRAS primer (A) and *galR* probe (B). (C and D) Northern blot analysis of RNA from *S. thermophilus* NZ302G and NZ302ΔR grown on glucose (Gl), lactose (L), or galactose (Ga) hybridized with the *galK* probe (C) and the *lacS* probe (D). The mRNA transcript sizes are indicated by arrows.

to those of NZ302G on all the sugars tested and thus were also induced on lactose and galactose.

LacZ activity was measured to determine the expression of the *lac* operon genes. In NZ302G, LacZ activity was induced about two- and fourfold during growth on lactose and galactose, respectively, in comparison to growth on glucose (Table 3). In contrast, LacZ levels in NZ302GΔR were not induced during growth on lactose. LacZ was again inducible in NZ302GΔR carrying the *galR*-expressing plasmid pNZ6811, although the activity was not as high as that of the original strain. Thus, the enzyme activity measurements confirmed the transcriptional analysis results, demonstrating the role of GalR as an activator of the *gal* and *lac* operons.

To analyze whether the activating role of GalR was specific for the galactose-utilizing strain NZ302G, the *galR* gene of *S. thermophilus* ST11, a well-characterized Lac^+ Gal^- strain (32), was also disrupted using the pNZ684 construct. PCR analysis indicated an identical organization of the *gal* genes in this strain and in CNRZ 302 (data not shown). Since strain ST11 grows very poorly on glucose, LacZ activities were compared using medium containing sucrose (Table 3). Both ST11 and ST11ΔR showed an induction of LacZ of approximately 2.5- to 3-fold after growth on lactose in comparison to growth on sucrose, but the levels of the LacZ activity of ST11ΔR were at least half those produced by ST11 on both carbon sources. The failure to fully induce LacZ in ST11ΔR strongly suggests that GalR also plays a role in activating the *lac* operon of this strain.

Characterization of the *galK* promoters for the Gal^+ mutants. To gain an understanding of the ability of NZ302G to transcribe the *gal* metabolic genes, in contrast to the parent strain CNRZ 302, a series of hybridization experiments using specific *galR* and *galK* probes on the *gal* region and PCR

TABLE 3. Enzyme activity measurements of *S. thermophilus* strains NZ302G, ST11, and derivatives

Carbohydrate	Strain	Gal-1-phosphate uridylyltransferase activity (nmol/min/mg of protein)[a]	β-Galactosidase activity (μmol/min/mg of protein)[a]
Glucose	NZ302G	59 ± 6	4.2 ± 0.9
	NZ302GΔR	19 ± 3	3.1 ± 0.5
	NZ302GΔR(pNZ6811)	70 ± 12	2.5 ± 0.4
Lactose	NZ302G	178 ± 73	7.7 ± 0.8
	NZ302GΔR	23 ± 9	3.1 ± 0.4
	NZ302GΔR(pNZ6811)	149 ± 35	6.7 ± 1.8
Galactose	NZ302G	230 ± 70	15.6 ± 2.8
	NZ302GΔR(pNZ6811)	393 ± 21	9.9 ± 0.8
Sucrose	ST11	ND[b]	4.8 ± 1.0
	ST11ΔR	ND	2.2 ± 0.3
Lactose	ST11	ND	13.3 ± 0.9
	ST11ΔR	ND	5.5 ± 0.6

[a] Results are expressed as mean ± standard deviation. The enzyme assays were performed on the same extracts within 4 h.
[b] ND, not determined.

amplification of the *galKTE* gene cluster were performed on the Gal$^+$ mutant. However, no DNA structural rearrangements were detected within *galR* or the metabolic *gal* gene cluster compared to the parent CNRZ 302 strain (data not shown). The *galR* and *galK* promoter regions of CNRZ 302 and NZ302G were amplified by PCR in duplicate with the same primers as those used in the primer extension experiments, and both strands of each product were sequenced. The analysis revealed that an extra A residue was inserted in a stretch of adenines preceding the −35 region (nt 1410 to 1414 [Fig. 4A]) of the *galK* promoter in the NZ302G DNA sequence, resulting in 6 A residues in the mutant in comparison to 5 in the Gal$^-$ parent. To determine whether other Gal$^+$ mutants of CNRZ 302 contained similar mutations in the promoter region, nine more independently isolated Gal$^+$ mutants of CNRZ 302 were investigated. DNA sequence analysis of these nine promoters showed that the *galK* promoter of each Gal$^+$ mutant also contained a point mutation. The mutants could be divided into three classes based on their mutations: class I consisted of five mutants with an A insertion, as described above for NZ302G; in class II, the three mutants contained a G-to-T substitution 3 bp preceding the −10 box; and the one mutant in the third class had a G-to-A substitution in the −10 box (Fig. 4A).

***galK* promoter activity.** The GalK activity of the mutant strains was used as a reporter for comparing the expression of the mutated promoters with that of the wild-type. A low level of GalK activity, 10 nmol/min/mg, was detected for the Gal$^-$ *S. thermophilus* CNRZ 302 grown on glucose-containing medium, and it increased to 37 nmol/min/mg on lactose. For the Gal$^+$ NZ302G and the other five class I mutants, the GalK activity increased from an average 46 nmol/min/mg on glucose to 264 and 305 nmol/min/mg on lactose and galactose, respectively. The activity increased from 53 nmol/min/mg on glucose to 193 and 323 nmol/min/mg on lactose and galactose, respectively, for the class III mutant. The highest activity was observed with the three class II mutants, an average of 68 nmol/min/mg on glucose to 400 and 458 nmol/min/mg on lactose and galactose, respectively. Thus, it is likely that the basal GalK activity has increased in the mutant strains due to promoter-up mutations, which allows sufficient expression of the *galK* gene on induction for galactose utilization.

To support our hypothesis that point mutations in the promoters were largely responsible for the Gal$^+$ phenotype of the mutants, the *galK* promoters of CNRZ 302 and SS2 (class II mutant) were cloned in front of the *gusA* reporter gene in plasmids pNZ6871 and pNZ6872, respectively. Both plasmids were stable in *E. coli*, but the frequency of transformation for pNZ6872 in *S. thermophilus* ST11 was consistently 10-fold lower than for pNZ6871. Since ST11 grows very poorly on glucose, the strength of the promoters was examined in medium containing sucrose or lactose. Expression of the *gusA* gene results in β-glucuronidase activity, which is indicated by the development of a blue color in colonies on plates containing the substrate X-Gluc. Surprisingly, growth of *S. thermophilus* ST11 transformants harboring pNZ6871 resulted in blue colonies on X-Gluc plates containing sucrose, while on lactose plates very small and bluer colonies developed, suggesting increased activity from the *galK* promoter. Colonies of ST11 harboring pNZ6872 developed as very small blue colonies on both sucrose and lactose plates, indicating strong activity of the class II mutant promoter on both carbon sources. However, ST11 transformed with pNZ6872 resulted in colonies that were smaller than those obtained with pNZ6871, and some white colonies also appeared. Analysis of the plasmid content of these colonies demonstrated that the pNZ6871 construct remained intact while deletion derivatives of the pNZ6872 construct were present in ST11 (data not shown). Thus, pNZ6872 is unstable, probably due to toxic effects of high β-glucuronidase activity.

DISCUSSION

This study demonstrates conclusively that *S. thermophilus* CNRZ 302 possesses the full complement of genes necessary for galactose metabolism despite its Gal$^-$ phenotype. In many organisms, the *galK, galT*, and *galE* genes, which constitute the Leloir pathway of galactose metabolism, may be clustered or organized in a single operon, and the order of these genes within the operon may be highly variable. While the similarity between the deduced primary sequences of the enzymes is very high within the lactic acid bacteria, the genomic organization of the *gal* clusters and gene order is species specific (17). The gene order for the *S. thermophilus galKTE* operon and the divergent *galR* gene is identical to that in *S. mutans* (2), which reflects the evolutionary relationship between these bacteria. A potential transcriptional regulatory gene, *galR*, has also been identified in *L. casei* and is transcribed in the *L. casei gal* operon *galKETRM* (6). In contrast, the putative homologues in *E. coli, galR* and *galS*, are not linked to the *gal* operon (56), and regulatory genes have as yet not been identified for other *gal* operons in lactic acid bacteria such as *L. lactis*, *Leuconostoc lactis*, and *Lactobacillus helveticus*.

The effect of glucose, lactose, and galactose on the *galR* mRNA levels strongly suggested that GalR was a transcriptional regulator of the *S. thermophilus gal* operon. Transcriptional analysis of the two *galR* copies generated following disruption of *galR* in the Gal$^+$ NZ302G indicated that the copy resulting in a C-terminally truncated GalR protein is driven by the *galR* promoter. The second copy, which lacks most of the DNA binding motif, was not transcribed. This was expected based on the orientation of the *galR* gene fragment in pNZ684. Since the substantial deletion from the C terminus includes residues contributing to inducer binding and dimerization (55), the truncated *S. thermophilus* GalR proteins are no longer functional. Northern analysis of NZ302GΔR confirmed that GalR functions as an activator of transcription for the *gal* operon and explained the inability of the disruption mutant to use galactose or the galactose moiety of lactose as a carbon source. The very low activity still detected for the GalT enzyme in NZ302GΔR might be due to a basal transcription level in the absence of GalR. Alternatively, it might reflect a low level of reversion to wild type. When the *galR* gene was provided in *trans*, the Gal$^+$ phenotype was restored.

The constitutive transcription of the nonfunctional *galR* gene in the absence of GalR indicates that the latter normally functions as a negative regulator of its own expression. Autoregulation is a common feature in prokaryotic gene regulation strategies. Negative autoregulation has been reported for other members of the LacI-GalR family such as GalS, PurR, and CytR (16, 30, 56). It is noteworthy that the majority of LacI-GalR members function as negative regulators while some

positive regulators also belong to this family and some (like CcpA) perform both functions (43, 45, 51).

The expression of the *lac* operon genes of both *S. thermophilus* NZ302G and ST11 is induced by growth on lactose- or galactose-containing medium. In both strains, β-galactosidase activity could no longer be induced to the usual level when the *galR* gene was disrupted, confirming that GalR also functions as a transcriptional activator of the *lac* operon. β-Galactosidase is more strongly induced in NZ302G in galactose-containing medium than in lactose-containing medium. This differential gene expression of the *lac* genes may be due to catabolite repression by the glucose moiety of lactose on the *lac* operon promoter (51), which will result in repression of the *lac* operon by lactose but not by galactose. Furthermore, the excretion of galactose by LacS in lactose medium would effectively reduce the availability of inducer in the cell.

The mutation to a Gal^+ phenotype does not result in constitutive expression of the *gal* genes that are induced in the presence of lactose and galactose, which strongly suggests that *S. thermophilus* was Gal^+ but became Gal^- in the recent past. While the advantages of the exchange reaction of the lactose transport protein offer a rationale for the observed excretion of galactose (40), the precise mechanism by which the enzymes of the Leloir pathway are suppressed has not been determined. Characterization of the 10 Gal^+ mutants of CNRZ 302 revealed that a point mutation had occurred in the *galK* promoter region of every isolate, the majority of which were single-base insertions in a homopolymeric run of adenine residues. Interestingly, a study of the molecular basis for the adaptive response of *E. coli* populations to conditions of nonlethal selection such as nutrient deprivation also identified single-base variations mainly in short mononucleotide repeats (14, 44), and slipped-strand mispairing was proposed as the responsible mechanism. In the *S. thermophilus galK* promoter, the G-to-A substitution in the class III mutant results in a −10 box (TACAAT) with greater homology to the −10 consensus (TATAAT) sequence (29). In class II mutants, the G-to-T substitution gives a TG doublet 1 bp upstream of the −10 sequence, which is a feature present in the promoters of gram-positive bacteria (12). This may correspond to the "extended −10" sequence that functions as a −35-independent promoter and requires the TG motif for efficient initiation at such promoters (24). Thus, these substitutions that resemble promoter-up mutations may increase the level of transcription of the *gal* genes and allow metabolism of galactose. The A insertion in class I mutants may also be a promoter-up mutation, although the reason for the enhanced activity in this case is not so apparent. The extra A increases the size of the inverted repeat preceding the −35 box from 11 to 15 nt (see below). In particular, the intrinsic DNA curvature that is predicted in this region is enhanced by the A insertion (data not shown), and this may result in increased promoter strength. The presence of curved DNA upstream of promoters, of which A tracts appear to be a major determinant, is associated with increased transcription (37).

The CNRZ 302 and SS2 *galK* promoter fusions to the *gusA* gene support the hypothesis that mutations in the *galK* promoter of *S. thermophilus* CNRZ 302 suppress the expression of the *gal* genes. Although β-glucuronidase activity was not expected from the ST11(pNZ6871) strain since galactokinase activity is barely detectable in CNRZ 302, factors such as the high copy number of this plasmid (12) and the gene dosage effect of the GalR activator are likely to be responsible. The only difference between the pNZ6871 and pNZ6872 plasmids was the G-to-T mutation in the *galK* promoter of the latter. Very high β-glucuronidase activity as a consequence of this promoter-up mutation would result in lethal effects on the host (P. G. G. A. de Ruyter, personal communication). This would explain the reduced frequency of transformation for pNZ6872, the small size of the blue colonies on medium containing X-Gluc, and the instability observed for this plasmid in the *S. thermophilus* host.

The intergenic region between *galR* and *galK* of *S. thermophilus* consists of 142 bp, which contains the promoter sequences of both genes in a back-to-back configuration (Fig. 4A). An 11-bp inverted-repeat sequence (IR) (nt 1392 to 1413; 5′-TTTTACTA-3′, 8 out of 11 matching nt) was detected in this region that could be an operator for GalR. The potentially global regulation by GalR prompted us to search for homologous sequences in the promoters of the *lac* operon and *galR* gene of CNRZ 302. Similar 11-bp IRs were found 13 bp upstream of the −35 box in the *lac* promoter and also overlapping the −10 box of the *galR* promoter (Fig. 4B). A consensus sequence could be deduced, with the 11-bp half of the IR consisting of a central 3 bp highly conserved portion, A(C/G)T, flanked on either side by four predominantly adenine and thymine bases. It is usually the −40 to −35 region of a promoter that is approached by an activator site; exceptions include the MerR family regulators (10, 35). In contrast, sites for repressors may be located from the −35 to −10 region. When activator proteins are used for repression, which generally occurs in cases of autoregulation, the operators often appear in positions for repression rather than activation. The potential operator sites for GalR conform to these general rules.

The *gal* genes of *S. mutans* are homologous to those of *S. thermophilus* and are organized in a similar divergent orientation (2). Alignment of the nucleotide sequences of the *S. thermophilus* and *S. mutans* promoter regions revealed homologous palindromic sequences, as described above (Fig. 4B). It is noteworthy, however, that a single-crossover disruption of the *S. mutans galR* gene resulted in constitutive expression of galactokinase, indicating that GalR functions as a repressor of the *gal* operon in this species. In contrast, GalR of *S. thermophilus* activates the *gal* and *lac* operons while repressing its own expression. The presence of these potential operators gives further credence to the hypothesis that repression of the *gal* operon was caused by recent mutations in the *galK* promoter.

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